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Development of human skin equivalents to unravel the impaired skin barrier in atopic dermatitis skin

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CHAPTER 2

Altered expression of epidermal lipid bio-synthesis enzymes in atopic dermatitis skin is accompanied by changes in stratum corneum lipid composition

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Abstract

The barrier dysfunction in atopic dermatitis (AD) skin is associated with stratum corneum (SC) lipid abnormalities including reduction of global lipid content, shorter ceramide (CER) as well as FFA chain length and reduced CER levels. However, the underlying causes of these changes has not been fully investigated. In the present study, we analyzed in AD patients and controls the expression of enzymes involved in the biosynthesis of free fatty acids (FFAs) and CERs in relation to the SC lipid composition.

These enzymes include β -glucocerebrosidase (GBA), acid-sphingomyelinase (aSmase), ceramide synthase 3 (CerS3) involved in CER synthesis and stearoyl CoA desaturase (SCD), elongase 1 (ELOVL1) and ELOVL6 involved in FFA synthesis. The results reveal an altered expression of SCD and ELOVL1 in AD lesional skin. This was accompanied by functional changes displayed by increased unsaturated FFAs (SCD) and reduced FFA C22-C28 (ELOVL1) in AD lesional skin. The CER composition in AD lesional skin showed corresponding changes such as increased CER AS and NS (aSmase) and decreased esterified ω -hydroxy CERs (CerS3). This study suggests that changes in the lipid composition in AD skin could arise from alterations in the expression of key enzymes in SC lipid synthesis and inflammation may influence the expression of these enzymes.

Introduction

Lesional and non-lesional skin in atopic dermatitis (AD) patients display inside-out barrier dysfunction (i.e. increased trans-epidermal water loss-TEWL) and outside-in impairments (increased penetration of compounds, such as allergens and pathogens through the skin) (1-4). Contributing factors to the development of AD include mutations in skin barrier related genes (e.g. filaggrin), skin inflammation and influences from the environment. The skin barrier is mainly located in the topmost layer of the skin the stratum corneum (SC). This layer is composed of corneocytes surrounded by a lipid matrix in a “brick” (corneocytes) and “mortar” (lipids) arrangement. The composition and organization of SC lipids is of utmost importance in maintaining the skin barrier. Cholesterol (CHOL), ceramides (CERs) and free fatty acids (FFAs) are the main lipid classes present in human SC. In healthy SC these are organized in lipid layers. Within these layers the lipids adopt predominantly a very dense orthorhombic lateral organization (5-9), see supplementary figure S1.

In AD lesional and non-lesional skin, the lipid abnormalities contributing to the barrier dysfunction arise from reduction in SC lipid levels in relation to protein content, reduced relative CER content (CER/CHOL ratio and CER levels) and a shorter mean lipid chain length. An increase in the fraction of unsaturated fatty acids has also been observed in lesional skin, while the fraction of hydroxy fatty acids is decreased (10-16). Particularly, the chain length reduction correlated excellently with a reduced skin barrier (17). The above mentioned lipid abnormalities in AD skin have been suggested to result from changes in the expression or activity of enzymes involved in SC lipid synthesis. The reduced lipid/protein ratio could originate from impairments in the secretion of lipids from lamellar bodies leading to an overall reduction of extracellular lipids (18, 19). Lipid synthesis enzymes such as β -glucocerebrosidase (GBA) and acid-sphingomyelinase (aSmase) catalyze the last step in CER subclass synthesis from glucosylceramides and sphingomyelin, respectively into CERs (20) and the activity of these enzymes depends on pH (21, 22). The increase in pH observed in AD skin may alter the activity and/or expression and degradation of GBA and aSmase and contribute to reduced CER content in AD and/or an imbalance in the level of CER subclasses (1, 23). Inflammatory cytokines upregulated in AD have also been shown to affect CER and FFA composition in *in vitro* studies (24, 25). In relation to the lipid chain length reduction observed in AD skin, enzymes which are necessary to synthesize the (ultra) long FFA chains (ELOVL1 and ELOVL4) or acylate the ultra-long FFA chains ($\geq$ C26) to the sphingoid backbone during biosynthesis of CERs (CER synthase 3 (CerS3)) may play a role (24). ELOVL1 elongates FFAs with carbon chain length of C20-C26 and ELOVL4 elongates those longer than C26 (26-29). In addition, ELOVL6 is primarily responsible for the elongation of FFAs with a chain length of C16 and C18. Changes in the expression of stearoyl CoA desaturase (SCD) which functions to produce unsaturated FFAs from saturated FFAs (30) may contribute to the increased level of unsaturated FFAs in AD (31).

This study aims to investigate whether the expression of CER and FFA biosynthesis enzymes are altered in AD skin compared with control skin. To determine whether changes in enzyme expression can be related with changes in lipid composition, we also examined lipid composition in the same group of patients and compared them with control skin. The expression of enzymes examined in this study include GBA, aSmase, CerS3, SCD, ELOVL1 and ELOVL6.

This study demonstrates that changes in the expression of CerS3, GBA, aSmase, SCD and ELOVL1 occur in AD skin and are more pronounced in lesional skin. The changes in protein expression in AD skin are accompanied by corresponding changes in lipid composition compared to control skin. The changes in lipid composition in SC of AD skin are in accordance with changes previously reported (15, 17).

Materials and methods

Study set-up and population

The study was approved by the ethical committee of the Leiden University Medical Centre and conducted according to the Declaration of Helsinki Principles after written informed consent from all the study subjects. Prior to the study, blood was collected in heparin coated tubes from study participants for isolation of DNA and genotyping. 8 Caucasian subjects without any history of dermatological diseases (24.3 ± 3.1 years; 5 female) and 20 Caucasian AD patients (27.0 ± 6.4 years; 12 female) were included. The AD study population consisted of 8 patients with filaggrin mutations (5 with heterozygous and 3 with homozygous *FLG* mutations) and 12 patients without filaggrin genotype mutations (see filaggrin mutation analysis below, results in supplementary table S1). The subjects did not apply dermatological or cosmetic products on their forearms one week prior to the study. The subjects were acclimatized for 45 minutes in a temperature and humidity controlled room after which the measurements were performed. All measurements were performed within a single day per subject, on the ventral forearms in the acclimatized room. The area of skin to be studied (non-lesional and lesional skin) was carefully selected by a dermatologist at the start of the study. At the selected areas, tape stripping, attenuated total reflectance fourier transformed infrared spectroscopy (ATR-FTIR) measurements were performed as described below. At the end of the study day, two 4mm biopsies were harvested from the ventral fore-arm of 7 patients close to the skin region used for the measurements (see below). This included one biopsy from non-lesional skin and one biopsy from lesional skin. On the study day, only 10 out of the 20 patients included in this study had lesional skin areas. From 3 control subjects, one biopsy was harvested from the ventral forearm on the study day.

SCORAD

In order to determine disease severity, local-SCORAD of non-lesional and lesional skin and overall SCORAD was determined by a dermatologist (see supplementary table S1) in the patient population according to the scoring method of Stalder and Taieb (32).

Immunofluorescence staining

The harvested biopsies were embedded in paraffin and cut at 5 μ m thickness. Immunohistochemical analysis of the following lipid biosynthesis enzymes was performed: GBA, aSmase, CerS3, SCD, ELOVL1 and ELOVL6. The primary and secondary antibodies are listed in supplementary table S2.

Immunofluorescence analysis was performed on both lesional and non-lesional biopsies from 7 AD patients compared to biopsies from 3 controls. Within this patient group, 3 of the patients showed heterozygous *FLG* mutation (R501X, 2282del4) and 4 patients a wild type filaggrin phenotype. The severity of AD measured by overall SCORAD ranged from 11.7 - 43.5. The sliced sections were stained as described previously (33, 34). Briefly, the sections were heat treated in sodium citrate buffer (pH 6), blocked with normal horse serum (Vector laboratories Burlingame, CA) and incubated with the primary antibody overnight at 4°C. Afterwards, sections were incubated with the appropriate secondary antibody (Rhodamine Red-X or

Cy3, Jackson immunoresearch Laboratory, USA) for 1 hour at room temperature and mounted using DAPI Vectashield (Vector laboratories Burlingame, CA).

Filaggrin mutation analysis

The controls and AD patients were screened for 4 of the most prevalent mutations observed in European Caucasians (R501X, R2447X, 2282del4, S3247X). The mutations were determined using blood samples and subsequent DNA isolation as described previously (35). In brief, using Ficoll density centrifugation, peripheral blood mononuclear cells (PBMCs) were isolated. The dry cell pellets were kept at -80°C until DNA isolation. Using the DNeasy Blood and Tissue kit (Qiagen, Venlo, Netherlands), DNA was isolated according to the manufacturer's instructions. Filaggrin mutation analysis revealed a patient distribution of 40% with mutations (25% heterozygous (R501X and 2282del4), and 15% homozygous (2282del4, S3247X)) and 60% wild type patients, see supplementary table S1.

Tape stripping procedure

Poly (phenylene sulfide) tape (Nichiban, Tokyo, Japan) was used to harvest SC lipids sequentially from control skin, lesional and non-lesional skin on the ventral fore arm (stripped area 4.5cm²). The tape was applied to the area of interest with a pressure of 450g/cm² using a D-squame pressure instrument (Cuderm Corp., Dallas, TX). The tape was removed in a fluent stroke with alternating directions for each successive strip. Each tape was punched into a circular area of 16mm in diameter and placed in a separate glass vial containing 1mL chloroform/methanol (2:1, all organic solvents were obtained from Biosolve BV, Netherlands) and stored at -20°C under argon until the lipids were extracted.

Lipid extraction

Lipids were extracted from tapes 6-9 of each study subject. A modified Bligh and Dyer extraction was performed on each tape separately (36, 37) as described previously (17). In brief, the tapes were incubated in 3 different ratios of chloroform: methanol: water (2:1:0; 1:1:0; 1:2:0.5) for one hour each. Afterwards, the extracts were pooled and a 0.25M KCl solution was added to the pooled extract. The extract was washed with water and the organic layer was collected after full separation of the water and organic layer. The final organic layer from the 4 tapes per subject was pooled and dried under N₂ gas at 40°C and reconstituted in 50µL chloroform: methanol: heptane (2.5:2.5:95). The samples were stored under argon at -20°C until analysis by liquid chromatography-mass spectrometry (LC-MS).

CER and FFA analysis by LC-MS

CER Species: 5 µl of each lipid sample (4 tapes/50µL, at 40°C) was injected and separated in an analytical normal phase column (PVA-bonded column; 100 x 2.1mm i.d., 5µm particle size, YMC (Kyoto, Japan)) under a flow rate of 0.8 ml/min and using a binary solvent system of heptane (100%, solvent A) and heptane/isopropanol/ethanol (50:25:25, solvent B) in an ACQUITY quaternary UPLC system (Waters, Milford, MA). The UPLC was coupled to a mass spectrometer (Xevo, TQ-S, Waters, Milford, MA) in atmospheric pressure chemical ionization (APCI; ion source II) and positive scan mode with a scan range of 350-1200 amu. The

temperature of the source heater and capillary voltage was set at 150°C and 3.5kV respectively. Deuterated CER NS (D47) was used as an internal standard. The CER analysis was performed using Target Lynx software version 4.1 and the nomenclature used throughout this article is according to Motta et al., and Masukawa et al., (38, 39). A detailed analyses of this method will be published elsewhere (Boiten et al., in preparation).

FFA species: 5 µl of each lipid sample (4 tapes/50µL, at 40°C) was injected and separated in a UPLC HSS T3 column (50 x 2.1mm i.d., 1.7µm particle size, Waters, Milford, MA) under a flow rate of 0.8 ml/min and using a binary solvent system of 0.5mM ammonium acetate in water (solvent A) and 0.5mM ammonium acetate in water/isopropanol/acetonitrile (5:70:25, solvent B) in the UPLC and mass spectrometer system described above. The UPLC was coupled to the mass spectrometer in electrospray ionisation (ESI), negative scan mode with a scan range of 200-600 amu. The temperature of the source heater and capillary voltage was set at 150°C and 1kV respectively. FFA Deuterated-C24 was used as an internal standard. The data was corrected for chain length and matrix effect which is described in the supplementary materials and methods.

Stratum corneum protein content

The Squamescan 850A (Heiland electronic, Wetzlar, Germany) was used to relate the absorption of the tape strip to the amount of protein removed by each strip. The poly (phenylene sulfide) tape was applied using a range of pressures between 0-450g/cm². This pressure range was used in order to vary the amount of stratum corneum removed per area. The absorption of 20 tape strips from 4 volunteers was measured by Squamescan and each tape strip was incubated overnight in 1mL of 1M KOH in 10X Hanks buffered saline solution (HBSS, life technologies Europe B.V., Bleiswijk, Netherlands). The pH of each tape strip solution was adjusted to 7/8 with 12M HCl. Thereafter, protein content was determined using Microplate BCA Protein Assay (Reducing Agent Compatible, Thermofischer scientific Inc., IL, USA) according to the manufacturers instruction. A calibration curve was obtained by plotting the protein level of each tape against the squamescan values measured at an optical density at 562nm. Using the calibration curve, the optical density at 562nm (squame scan) from each tape strip of the patients and controls was calculated.

Lateral organization and conformational order of SC lipids

ATR-FTIR spectroscopy was utilized to analyze the lateral organization and conformational order of the lipids in all study subjects. FTIR spectra of the SC (from the same location as used for analysis of lipid composition) were collected as a function of depth after two tape stripping's using a Varian 670-IR spectrometer (Agilent technologies, Santa Clara, CA) until 12 tape strips were collected. The spectrometer was equipped with i) a broad band mercury- cadmium-telluride (MCT) detector and ii) an external sample compartment containing an attenuated total reflection (ATR) accessory (GladiATR, Pike, Maddison, WI) having a single reflection diamond. The sample compartment was constantly purged with dry air. Each spectrum was collected as an average of 200 scans at a resolution of 2cm⁻¹. The peak position of the CH₂ symmetric stretching vibration and the band width from the second derivative CH₂ scissoring region was determined as described previously (8, 40) using Resolutions Pro 4.1 software (Agilent technologies, Santa Clara, CA).

The scissoring bandwidth was calculated at 50% of the maximum peak height. An increase in width of this band indicates that more lipids adopt a dense lipid organization (an orthorhombic lateral packing). The position of the CH₂ symmetric stretching vibration was also determined as a measure for the conformational disordering of the lipids. A high value of the stretching vibrations indicates a liquid phase, whereas a value <2850 cm⁻¹ indicates a crystalline phase. Mean values of 5 measurements were calculated per subject for each parameter (measurements were performed using tape strips 2-10 so as to analyze lipids within the same depth as with the lipid composition analysis).

Data analysis

Statistical analysis was performed with GraphPad prism 5 (GraphPad software, CA, USA). When comparing two groups, non-parametric Mann-Whitney tests were performed and $p < 0.05$ indicates statistical significant data (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). In order to analyze the correlation between two parameters, a bivariate analysis was performed and the Spearman's ρ correlation coefficient was calculated. In total 7 patients from the AD patient population and 3 controls were selected for immunofluorescent analysis (supplementary table S3). This subset of subjects representatively reflect the range of AD severity of the entire study population. The overall SCORAD from this AD patient subpopulation ranged from low (11.7-25.8) to high (29.8-43.5), whereas SCORAD of controls was always 0.

Results

Expression of SCD and ELOVL1 is altered in AD lesional skin

In order to investigate the underlying factors that may contribute to the change in barrier properties in AD skin, we analyzed the expression of specific enzymes involved in the biosynthesis of the lipids in SC and related the expression of these enzymes with lipid composition. Stearoyl CoA desaturase (SCD) is an important enzyme in the formation of unsaturated FFAs from saturated FFAs. In control skin, SCD is only expressed in the stratum basale, while in AD non-lesional skin it is expressed in the stratum basale and in 1-2 lower layers of the stratum spinosum. Lesional AD skin shows SCD expression in more epidermal cell layers (figure 1, see supplementary figure S2 for images without DAPI staining). This change in SCD expression is observed in all AD biopsies analyzed.

The elongation of FFAs is an important step in the process of SC FFA synthesis. FFA elongases such as ELOVL1 and ELOVL6 are involved in this process. Specifically, ELOVL1 is an important enzyme in elongation of FFAs with chain lengths ranging from C20-C26. The expression of ELOVL1 in control and AD non-lesional skin is visualized as a gradient from stratum basale to stratum granulosum with the most pronounced expression in the granular and upper spinous layer. However, in the majority of the biopsies in AD lesional skin (4 out of 7), this gradient in ELOVL1 expression is lost and the expression of ELOVL1 was reduced and equal across the epidermal layers (figure 1). ELOVL6 which elongates C16 to C18 also shows a gradient expression in the epidermis with the highest expression in the stratum granulosum in control skin. The expression of ELOVL6 remained unchanged in all AD lesional and non-lesional skin biopsies (figure 1). No differences in the expression of SCD, ELOVL1 and ELOVL6 were noticed between patients with flag-grin mutations (homozygous and heterozygous) and wild type patients (data not shown).

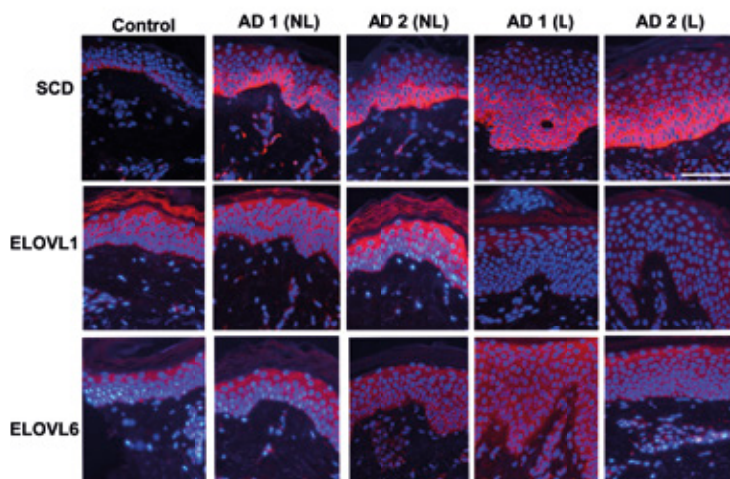


Figure 1: Expression of SCD and ELOVL1 are changed in AD lesional skin.

Immuno-histochemical staining of stearyl CoA desaturase (SCD), ELOVL1 and ELOVL6 in one control, non-lesional and the corresponding lesional skin from two AD patients. SCD is expressed in the stratum basale in control skin and extends to suprabasal layers of the epidermis in AD non-lesional and to even more epidermal cell layers in lesional skin. ELOVL1 in control and AD non-lesional skin is visualized as a gradient from stratum basale to stratum granulosum with the most pronounced expression in the granular and upper spinous layer. In most patients, ELOVL1 expression was reduced and equal across the epidermal layers in lesional skin. ELOVL6 also shows a gradient expression in the epidermis with the highest expression in the stratum granulosum in control skin. This remained unchanged in all AD lesional and non-lesional skin biopsies. Representative images are shown with 20x magnification, scale bar 50µm. The overall SCORAD of AD 1 images is 25.8 and AD2 (non-lesional and lesional) 41.3. NL: non-lesional, L: lesional. See supplementary figure S2 for images without DAPI staining.

Different expression pattern of late stage CER synthesis enzymes in AD lesional skin

The expression of CerS3, aSmase and GBA was also analyzed by immunofluorescence staining. Firstly, the highest expression of CerS3 is observed as a one cell layer band along the stratum granulosum in all control and AD non-lesional biopsies with a weaker expression in deeper epidermal layers. Its function has been linked to the production of ultra-long chain CERs with acyl chain lengths of C26 and above (29). The highest expression pattern of CerS3 in AD lesional skin is seen in the 2-4 upper cell layers in various sections along the entire biopsy. This occurred in 5 out of 7 of the biopsies analyzed (figure 2, see supplementary figure S3 for images without DAPI staining). Secondly, aSmase which is involved in the synthesis of CER AS and CER NS (20, 41) shows a gradient expression through the epidermis with the highest expression in the stratum granulosum in control and non-lesional AD skin (2 cell layers, figure 2). In AD lesional skin, the gradient in expression of aSmase is lost as the expression of aSmase is very similar in all epidermal layers. The changes observed in aSmase expression occurred in all analyzed AD biopsies. Thirdly, the GBA expression in control human skin is at the interface between the stratum granulosum and SC and hardly no staining occurs in

the deeper epidermal layers. In AD non-lesional and lesional skin of patients with low SCORAD (overall SCORAD: 11.7-25.8; local SCORAD: 3-6), no differences were observed in the expression of GBA as the expression was similar to control (figure 2, left lesional image). However, in patients with higher SCORAD (4 patients, overall SCORAD: 31.8-43.5; local SCORAD: 6-8), GBA was expressed in 2-3 cell layers along the stratum granulosum and stratum spinosum in lesional skin (figure 2, right lesional image). The expression of GBA in AD non-lesional skin with high SCORAD was also similar to the control skin (figure 2, left non-lesional image). In addition, these changes in GBA, aSmase and CerS3 expression were not affected by *FLG* mutations as the reported changes were observed in both wild type AD patients and patients with homozygous and heterozygous *FLG* mutations (data not shown).

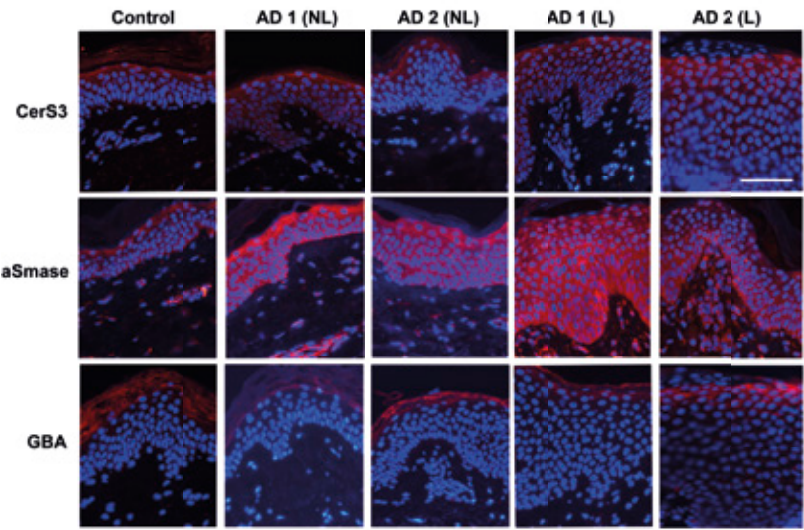


Figure 2: Expression of CerS3, aSmase and GBA are changed in AD-lesional skin. Immuno-histochemical staining of CER synthase 3 (CerS3), acid-sphingomyelinase (aSmase) and glucocerebrosidase (GBA) in control, atopic dermatitis (AD) non-lesional and corresponding lesional skin. The highest expression of CerS3 is observed as a one cell layer band along the upper stratum granulosum cell layer and a weaker expression in deeper epidermal layers in all control and AD non-lesional biopsies. The highest expression extended to 2-4 cell layers in the epidermis in lesional AD skin. aSmase also shows a gradient expression which peaks at the stratum granulosum in control and non-lesional AD skin. The gradient expression is no longer present in lesional AD skin as aSmase is similarly expressed in the entire epidermis. GBA is expressed in control and non-lesional AD skin at the interface between the stratum granulosum and SC. Changes in GBA expression was only observed in lesional skin of patients with overall SCORAD ranging between 31.8-43.5 where GBA expression was extended to 2-3 cell layers along the stratum granulosum and stratum spinosum. Representative images are shown with 20x magnification, scale bar 50µm. The overall SCORAD of the left AD images (non-lesional and lesional) ranges from 11.7-25.8 and for the right AD images (non-lesional and lesional) 31.8-43.5. NL: non-lesional, L: lesional. See supplementary figure S3 for images without DAPI staining.

Changes in the expression of FFA bio-synthesis enzymes is accompanied by changes in FFA composition in AD skin

To relate the changes in enzyme expression with the consequences at a functional level, the CER and FFA composition was analyzed by LC-MS. The amount of SC removed (total protein content) from both control and AD skin was determined to examine whether the lipids analyzed were harvested at similar depths in SC. There was no significant difference between the amount of SC removed between control and non-lesional and lesional AD skin (data not shown).

When we focus on the degree of unsaturation of the FFA, in comparison with control skin, non-lesional AD skin showed no significant change in the level of unsaturated FFAs ($p=0.16$), although a higher expression of SCD was observed in non-lesional skin. In AD lesional skin, a significant increase in unsaturated FFAs was observed compared with control skin ($p<0.01$, Figure 3a). This may coincide with the higher expression of SCD observed in lesional skin.

The FFA chain length distribution was also examined with respect to the FFA specifically the chain length distribution of both saturated and unsaturated FFA are shown in supplementary figure S4.

Since ELOVL1 is particularly involved in the elongation of FFA with chain length of C20-C26 we calculated the total level of FFA C22-C28 from the chain length distributions (saturated and unsaturated). No significant difference was observed in the levels of FFA C22-C28 in AD non-lesional skin ($p=0.27$). However, there was a decrease in the FFAs C22-C28 in AD lesional skin in relation to control skin ($p<0.05$, Figure 3b). As ELOVL4 is an important enzyme to elongate the ultra-long FFA chain from C26 and longer, we also calculated the FFAs $\geq C26$ and observed a similar trend: The level of FFAs $\geq C26$ were significantly reduced in AD lesional skin compared to control skin ($p=0.04$, Figure 3c). FFAs with chain length of C16-C18 (saturated and unsaturated) was significantly increased in AD lesional skin ($p<0.05$, figure 3d) compared to control skin.

As previous *in vitro* studies indicated that specific changes in FFA composition contributes to a less tight lateral packing in the SC, the lipid organization in the SC was examined using ATR-FTIR (31). To obtain this information we focused on the CH_2 scissoring and the CH_2 symmetric stretching vibrations of the lipid hydrocarbon chains. These vibrations provide information on the density of the lipid packing and the conformational disordering of the lipids respectively. An increase in width of the CH_2 scissoring band width at half maximum in the infrared spectrum indicates that more lipids adopt a dense lipid organization (an orthorhombic lateral packing) (40). A high value of the CH_2 symmetric stretching vibrations ($>2852\text{ cm}^{-1}$) indicates a liquid phase, while a value $<2850\text{ cm}^{-1}$ indicates an ordered phase (42, 43). The lipids in AD non-lesional and lesional skin showed less ordering as the CH_2 symmetric stretching vibrations of the lipid hydrocarbon chains was significantly increased in relation to control lipid organisation ($p<0.001$, supplementary figure S5a). Similarly, the lipids present in AD lesional skin also show a less dense lateral packing compared to control skin as the CH_2 scissoring band width was significantly reduced in both groups compared to the control ($p<0.01$, supplementary figure S5).

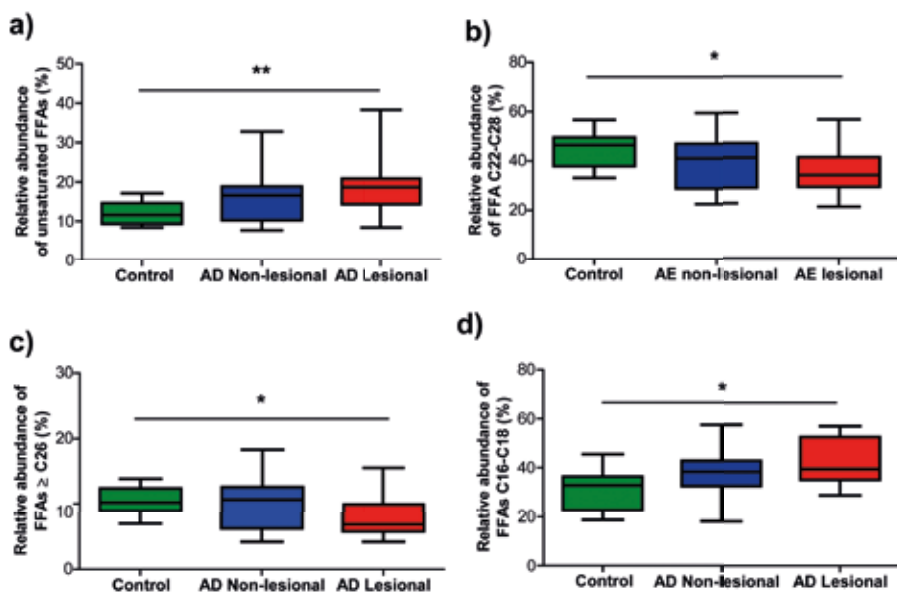


Figure 3: Changes in FFA composition in control, lesional and non-lesional AD skin.

A) Box plots (Whiskers: min to max) showing the relative level of unsaturated FFA. Unsaturated FFAs are significantly increased in AD lesional skin. B) Box plots illustrating relative abundance of FFA with chain length of C22-C28 (saturated and mono-unsaturated). These FFAs were significantly decreased in AD lesional skin. C) Illustrates the level of FFA with chain length $\geq$ C26 (saturated and unsaturated) which is also significantly reduced in AD lesional skin. D) The relative abundance of FFA with chain length C16-C18 (saturated and unsaturated) as illustrated by box-plots is significantly increased in AD lesional skin

Changes in CER synthesis enzyme expression are accompanied by changes in CER composition in AD skin

12 subclasses of CERs in human SC are derived from the linkage of 3 possible fatty acid chains (non-hydroxy fatty acid [N], α -hydroxy fatty acid [A] and esterified ω -hydroxy fatty acid [EO]) to 4 different sphingoid bases (dihydrosphingosine [dS], sphingosine [S], phytosphingosine [P], 6-hydroxysphingosine [H]). The nomenclature of CERs described above is illustrated in supplementary figure S6.

As a gradient of the expression of aSmase was not observed in lesional skin, we determined the levels of CER [NS] and [AS], since aSmase catalyzes an important final step in the synthesis of these CER subclasses. The levels were not significantly increased in AD non-lesional skin however, a significant increase in the level of CER [NS] and [AS] was observed in AD lesional skin ($p < 0.05$, figure 4a).

Since we observed changes in expression of CerS3 that is particularly important for the synthesis of the ultra-long chain CERs, the level of [EO] CERs (EOS, EOP, EOdS, and EOH) was also investigated. [EO] CERs are known to be important for the skin barrier function and in our cohort accounts for $7.17 \pm 2.06\%$ of CERs

in control skin. This is significantly decreased to $5.20 \pm 1.87\%$ in AD lesional and $5.15 \pm 1.70\%$ in non-lesional skin ($p < 0.05$, figure 4b). It may be clear that no significant differences in [EO] CER level was observed between lesional and non-lesional AD skin. As the FFAs with a chain length of C16 and C18 was increased and a common synthetic pathway of CERs and FFAs has been demonstrated in a previous study, we also examined whether the fraction of CERs with a very short chain length (CER C34) was increased (17). The fraction of CER C34 in lesional skin was $1.67 \pm 1.06\%$ which was significantly higher than in control skin ($0.35 \pm 0.27\%$, $p < 0.001$, data not shown). When comparing phytosphingosine based CERs with sphingosine based CERs we also observed a highly significant correlation ($r = -0.76$, $p < 0.0001$, Figure 4c). This result suggests that a relative increase in the sphingosine CER subclasses observed in AD skin is at the expense of the CER classes with a phytosphingosine base. No significant change was noticed in the level of CERs with a dihydrosphingosine or 6-hydroxy sphingosine base.

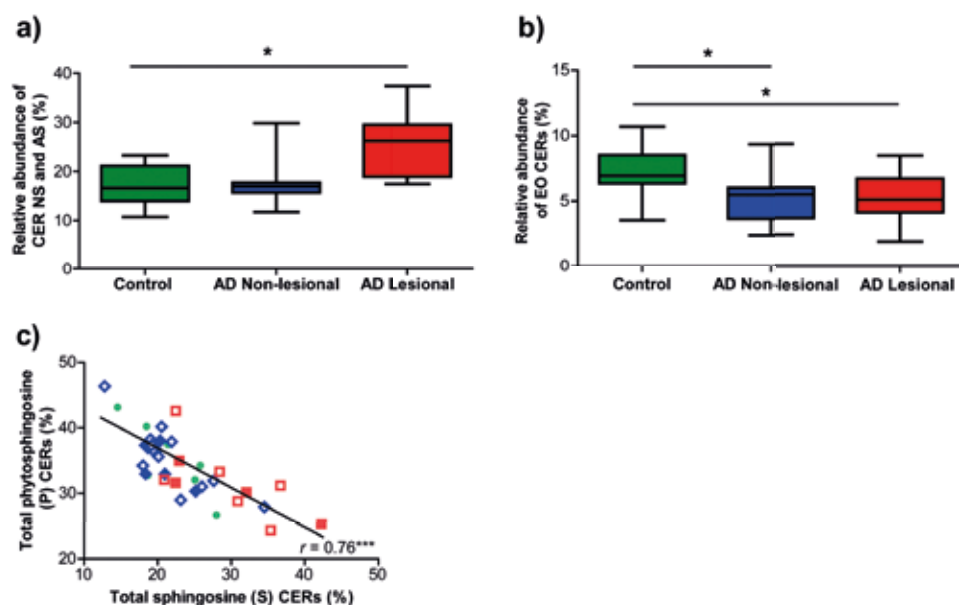


Figure 4: Changes in CER composition in AD skin.

A) Relative level of CER [AS] and [NS] is significantly increased in lesional skin in relation to control skin. B) Levels of [EO] CERs in control, AD lesional and non-lesional skin. [EO] CERs are significantly decreased in both lesional and non-lesional AD skin. C) Correlation plot of relative level of phytosphingosine CERs versus sphingosine CERs which shows a positive spearman correlation. The spearman correlation coefficient is displayed in the lower right corner. Control skin is indicated by ●, AD non-lesional skin by ◇ and AD lesional skin by □. The values from patients with filaggrin mutations and wild type are represented by filled symbols and open symbols respectively.

Discussion

This study analyses the expression of enzymes involved in the biosynthesis of FFAs and CERs in relation to their final product in SC of AD skin. We especially focused on 3 enzymes involved in FFA synthesis: SCD, ELOVL1 and ELOVL6 and 3 enzymes involved in CER synthesis i.e. GBA, aSmase and CerS3 (see supplementary figure S7).

Lipid synthesis and inflammation

In a previous study focusing on lesional and non-lesional skin of AD patients, it was shown that the average SC lipid chain length in each patient in lesional skin was reduced compared to non-lesional skin in the same patient. This was caused by a reduction in average chain length of both CERs and FFAs. Furthermore, the level of [EO] CER subclasses was also reduced.

In this work we observe that overall changes in the expression of the enzymes were more pronounced in lesional skin than in non-lesional skin in each of the patients analysed and that changes in enzyme expression correspond with the changes in lipid composition. Although the reported changes in lipid composition are all relative, the changes in enzyme expression were in most cases associated with changes in lipid composition in the SC. As clear differences between lesional and non-lesional skin are noticed, the expression of enzymes involved in lipid metabolism may not only be dependent on genetic background of the patients, but inflammation and environmental factors (e.g. dry air, stress, using soaps, pH) may also play an important role (24, 25, 44-46). AD skin lesions are associated with increased levels of T_H1 , T_H2 and T_H17 cytokines (47). IL-4 has been proposed to inhibit barrier homeostasis as it reduces the mRNA levels of aSmase and GBA. It also reduces the CER levels in barrier disrupted human skin equivalents (48). In addition, IFN- γ reduced the mRNA expression of ELOVLs and CER synthases in cultured human keratinocytes and in epidermal sheets (24). The CER [NS] chain length was analysed in detail and it was shown that after supplementation of IFN- γ the levels of longer chain CER [NS] were more drastically reduced than those of the shorter CER [NS] (24). Other cytokines such as IL-31 and TNF- α alter the protein expression of ELOVL1 and CerS3 and reduce the level of EO CERs and FFAs with chain length $\geq C24$ (24, 46).

Enzymes involved in FFA synthesis

In AD lesional skin, the expression of SCD is observed in an increased number of viable cell layers in the epidermis. SCD-1 plays a crucial role in the biosynthesis of unsaturated FFAs C16:1 and C18:1 (26). We observe a significant increase in unsaturated FFAs in AD lesional skin. This suggests that the increase in SCD expression may have a functional effect on the FFA composition and is responsible for the increased level of unsaturated fatty acids. However, changes in the enzymatic activity of SCD cannot be excluded as a contributing factor. *In vitro* studies using lipid membranes demonstrate that higher levels of unsaturated FFAs contribute to a higher level of conformational disordering and an increase in the level of lipids forming a hexagonal lateral packing (31). Therefore the increased degree of FFA unsaturation and reduction in FFA chain length may contribute to the reduced density of packing and increase in conformational disordering in the lipid matrix of AD skin (17).

The distribution of ELOVL1 expression clearly differs in AD lesional skin. As ELOVL1 elongates FFA with chain length between C20-C26, the change in its expression may contribute to the reduction in FFAs C22-C28 observed in AD lesional skin. We also observe a similar trend in the level of FFAs (SFA and unsaturated FFA) $\geq$ C26 produced by ELOVL4. These very long FFAs were significantly reduced in AD lesional skin compared to control subjects. This indicates that the reduction in level of FFAs C22-C28 is probably not influenced by a higher activity and/or expression of ELOVL4. Unfortunately, the expression of ELOVL4 could not be examined as no suitable antibodies were available. The expression of ELOVL6 which contributes to the biosynthesis of FFA C16 and FFA C18 is unaffected in AD skin (17). However, there is an increase in these short chain FFAs (unsaturated and saturated) in AD lesional skin. The level of FFAs can be influenced by elongases (particularly ELOVL1) and CerS which utilise FFAs in the synthesis of CERs. As there is an increase in the short chain CERs (i.e. CER C34 and CER C36), this accumulation of FFA C16 and C18 may not be due to a reduced expression or activity of ceramide synthases responsible for n-acylating sphingoid bases to FFA C16 and FFA C18 (49). Reduced ELOVL1 expression which is accompanied by reduced FFA C22-C28 may therefore be one of the underlying factors for accumulation of short chain FFA C16 and FFA C18, mainly being the unsaturated FFAs. The expression of CerS3 which n-acylates sphingoid bases to very long chain FFA ($\geq$ C26) was only affected in AD lesional skin. In relation to CerS3 expression, a significant reduction in [EO] CERs was observed in AD lesional and non-lesional skin in agreement with previous studies (15, 50). The level of very long chain FFA ($\geq$ C26) between non-lesional and lesional skin was not statistically different. This may contribute to the absence of a significant difference in [EO] CER levels between non-lesional and lesional skin.

Enzymes involved in CER synthesis

When we focus on enzymes involved in the last step of biosynthesis of CERs, we observe that the gradient in expression of aSmase in the epidermis with a stronger expression in stratum granulosum is lost in lesional skin. As an increased level of CER [AS] + CER [NS] was observed, a reduced expression of aSmase in lesional skin was not expected since one of the precursors is sphingomyelin. The abundance of CER [AS] + CER [NS] relative to the abundance of the remaining CER subclasses depends on the relative expression and enzyme activity of GBA and aSmase (51). It has been reported that there is an increase in the pH in non-lesional and lesional AD skin (20, 52), and the optimal pH for the activity of GBA and aSmase are 5.2 and 4.5-5, respectively. Therefore, the activity of both GBA and aSmase may be affected in AD skin (21, 53, 54). For aSmase this has been reported (1) and previous studies have shown no change in GBA activity in AD non-lesional SC (55, 56). However, this is not reported for AD lesional skin where the most drastic changes in CER profile are observed. As the expression of GBA is more localized at the interface between the viable epidermis and the stratum corneum, while the expression of aSmase is also present in deeper epidermal layers, the activity of the GBA may be more affected by an increased pH than that of aSmase. This would result in a higher relative level of CER [NS] + CER [AS], as sphingomyelin is only a precursor of CER [NS] and CER [AS] which is what is observed in AD lesional skin. The level of [P] based CERs and [S] based CERs showed a very significant spearman correlation. Since [S] based CERs (NS and AS) are increased in AD lesional skin, the results suggest a relative feedback mechanism between the syntheses of [S] and [P] CERs in AD skin however this requires further analysis beyond the scope of this study.

Even though the changes in CER composition and lipid chain length described in this study and from previous studies are represented as a relative values, similar results were observed in studies which analyse the absolute amounts of lipids in AD skin (10, 12, 57). This further emphasises a relationship between lipid composition and lipid enzymes expression described in this study.

In conclusion, this study points out two major findings. Firstly, the changes in the lipid composition in AD skin could partly arise from changes in the expression of key enzymes in SC lipid synthesis such as SCD, ELOVL1, aSmase, GBA and CerS3. Secondly, inflammation seems to influence the expression of these enzymes as the changes in expression are mainly observed in AD lesional skin.

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Supplementary Figures

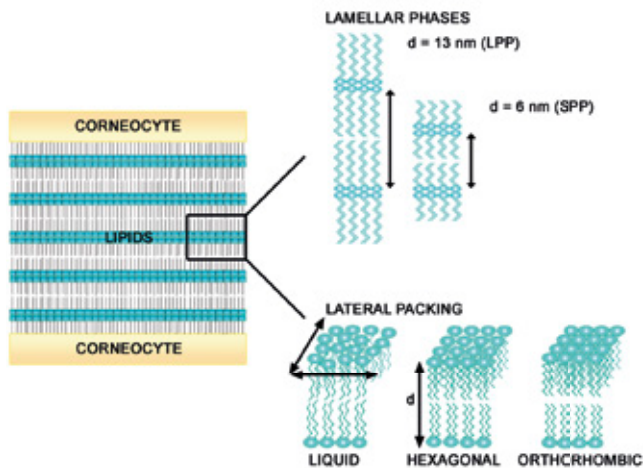


Figure S1: Lipid organization in human stratum corneum

The stratum corneum (SC) is the topmost layer of the epidermis which comprises of corneocytes surrounded by an organized intercellular lipid matrix. In the lateral lipid organization, the lipids can be arranged in an orthorhombic (very dense and ordered), liquid (disordered) and hexagonal (less dense and ordered) organization. (Adapted from Thakorsing et al., (1))

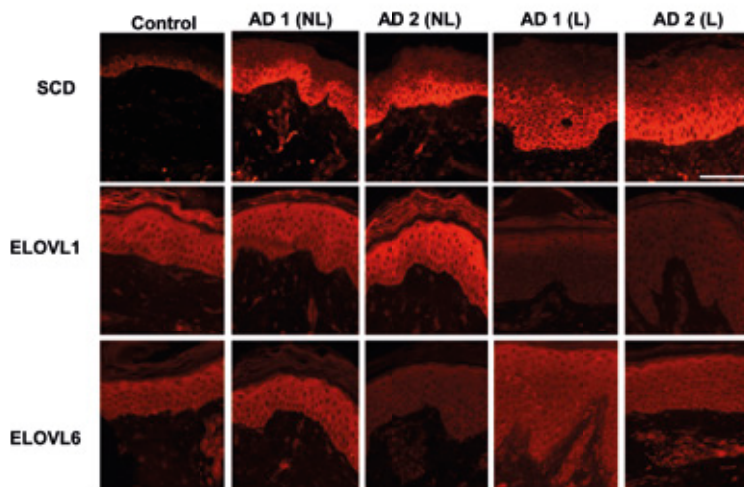


Figure S2: Expression of SCD and ELOVL1 are changed in AD lesional skin.

Immunohistochemical staining of stearyl CoA desaturase (SCD), ELOVL1 and ELOVL6 in one control, non-lesional and the corresponding lesional skin from two AD patients (as shown in figure 1) *without DAPI* staining. Representative images are shown with 20x magnification, scale bar 50µm. NL: non-lesional, L: lesional.

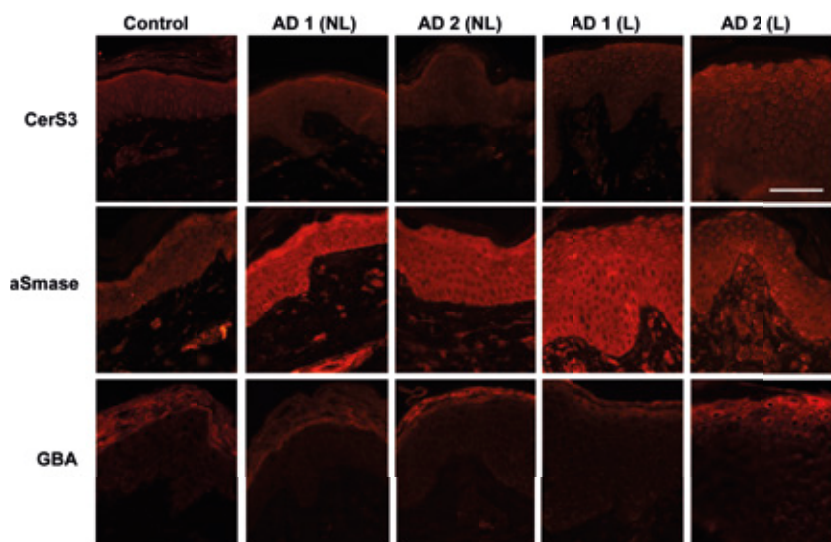


Figure S3: Expression of CerS3, aSmase and GBA are changed in AD-lesional skin.

Immuno-histochemical staining of CER synthase 3 (CerS3), acid-sphingomyelinase (aSmase) and glucocerebrosidase (GBA) in control, atopic dermatitis (AD) non-lesional and corresponding lesional skin (as shown in figure 2) *without DAPI* staining. Representative images are shown with 20x magnification, scale bar 50µm. NL: non-lesional, L: lesional.

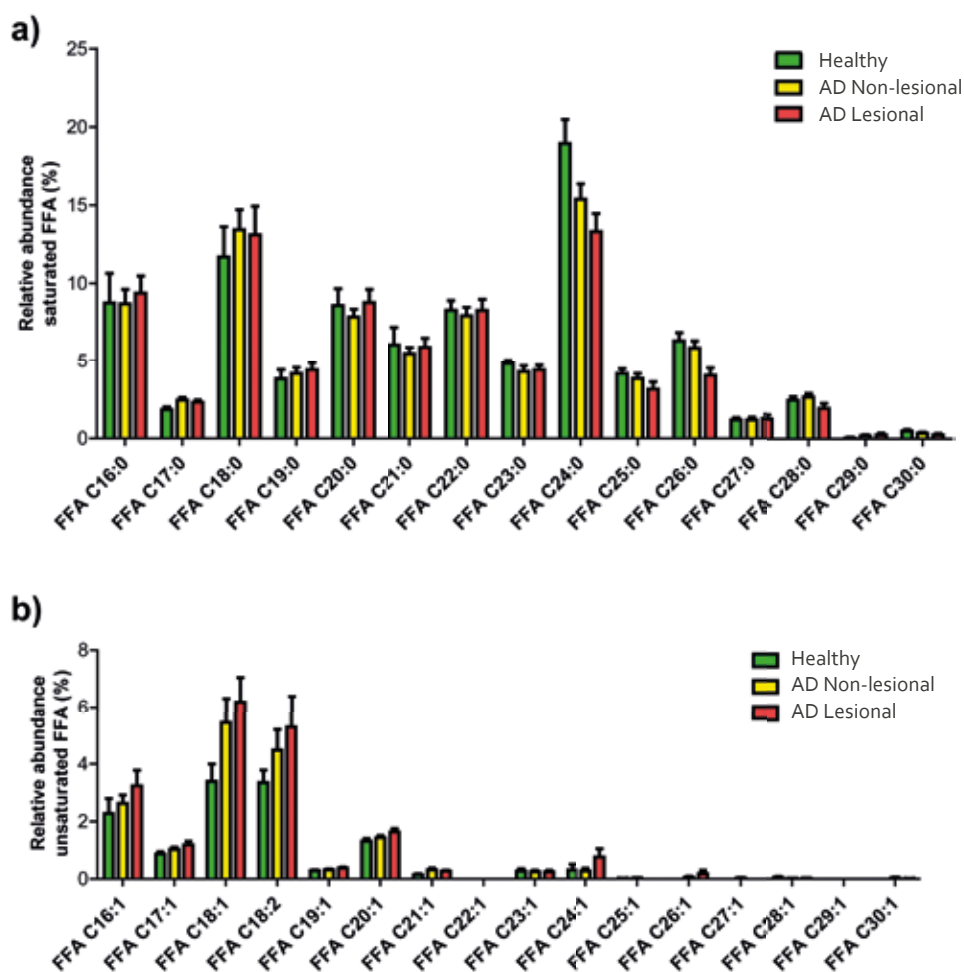


Figure S4: Relative abundance of FFA subclasses (saturated and unsaturated)

a) Bar plots showing the chain length distribution of saturated free fatty acids (FFAs) and b) unsaturated FFAs in healthy, AD non-lesional and AD lesional skin. The total amount of all FFAs was set to 100%. There is a significant trend in the decreasing abundance of saturated FFA C24:0- FFA C30:0 in lesional AD skin ($p < 0.0001$). In the chain length distribution of unsaturated FFA, there is a trend wise increase in the level of unsaturated FFA C16:1- FFA C30:1 in non-lesional and lesional AD skin ($p < 0.05$ and $p < 0.001$ respectively).

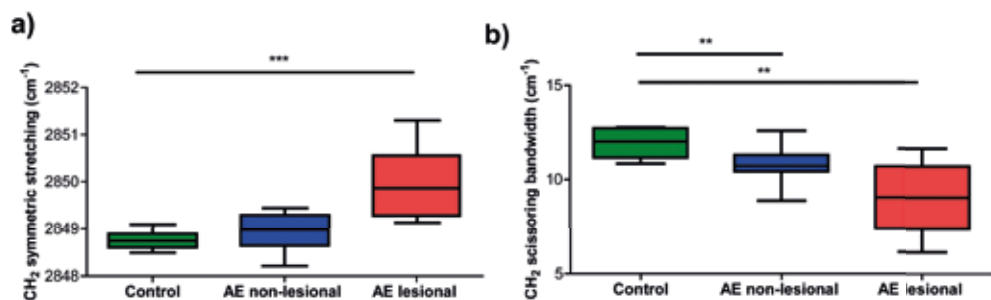


Figure S5: Lipid organization parameters in AD patients and control subjects

A) Position of the CH₂ symmetric stretching vibration. The lipids present in AD lesional skin are less ordered than in non-lesional and control skin as shown by a significant increase in the CH₂ symmetric stretching vibrations of the lipid hydrocarbon chains ($p<0.001$). B) CH₂ scissoring bandwidth in the FTIR spectrum. AD non-lesional and lesional skin show less dense lateral packing compared to control skin as the CH₂ scissoring band width was significantly reduced in both groups compared to the control ($p<0.01$). *** $p<0.001$, ** $p<0.01$.

Fatty acid			
	Non hydroxy fatty acid (N)	α -hydroxy fatty acid (A)	esterified ω -hydroxy fatty acid (EO)
Sphingoid			
Dihydrosphingosine (dS)	NdS	AdS	EOdS
Sphingosine (S)	NS	AS	EOS
Phytosphingosine (P)	NP	AP	EOP
6-hydroxy sphingosine (H)	NH	AH	EOH

Figure S6: Nomenclature of stratum corneum ceramides

The ceramides in human stratum corneum comprise of one sphingoid bases which is either i) dihydrosphingosine (dS) ii) sphingosine (S) iii) phytosphingosine (P) or iv) 6-hydroxy sphingosine (H)) chemically linked to a fatty acid chain. The fatty acids are i) a non-hydroxy fatty acid (N), ii) an α -hydroxy fatty acid (A) or iii) esterified ω -hydroxy fatty acid (EO)). These result 12 ceramide subclasses in human stratum corneum.

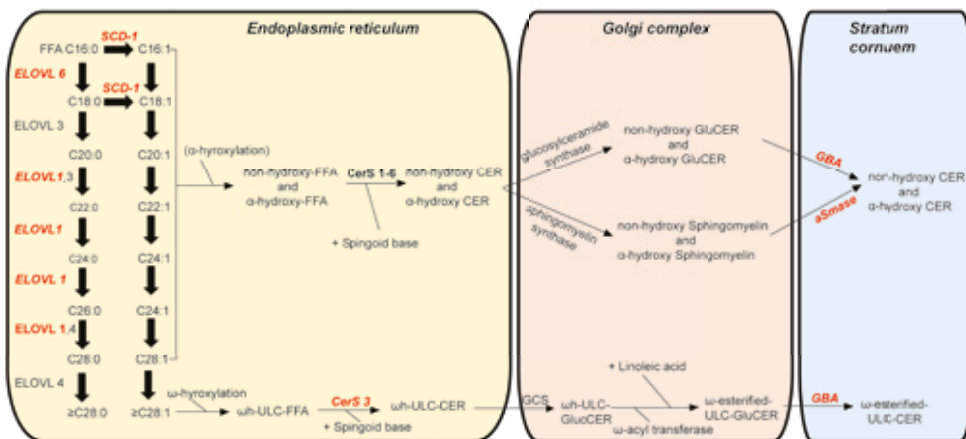


Figure S7: Illustration of late stage free fatty acids and ceramide synthesis

ELOVL: elongase, CerS: ceramide synthase, GBA: glucosylcerbrosidase, aSmase: acid-sphingomyelinase, SCD: stearyl CoA desaturase, wh: omega-hydroxy, GCS: Glucosylceramide synthase. The enzymes investigated in this study are highlighted in red (2-6).

Supplementary tables

Table S1: Local, overall SCORAD and filaggrin mutations of AE patients

SCORAD				Filaggrin Mutation			
Patient no.	local (non-lesional)	local (lesional)	Overall SCORAD	Wild type	R501X	2282del4	S2347X
1	1	3	25.8	+/+			
2	1	6	32.9	+/+			
3		-			+/-		
4	2	7	43.5			+/-	
5	0	2	29.8	+/+			
6	0	4	14.8	+/+			
7	0	-	10.9	+/+			
8	1	-	14.8	+/+			
9	0	-	8.2	+/+			
10	0	-	14.8	+/+			
11	0	-	0	+/+			
12	1	3	23	+/+			
13	0	4	23.4		+/-		
14	1	8	31.8	+/+			
15	0	-	0	+/+			
16	0	-	18.7			-/-	
17	1	-	24.9		-/-		
18	2	-	17.9			-/-	
19	0	4	11.7				+/-
20	2	6	41.3		+/-		

No. of patients with non-lesional areas: 20, No. of patients with lesional areas: 10

Table S2: Primary and secondary antibodies for immunohistochemical staining

Antibodies	Clone	Dilution	Company
Primary antibodies			
Rabbit SCD	Polyclonal	1:100	Sigma-Aldrich
Rabbit acid-sphingomyelinase	Polyclonal	1:1000	Abcam, UK
Mouse β -glucosylcerebrosidase	IgG2a	1:400	Abcam, UK
Rabbit ELOVL1	Polyclonal	1:300	Santa Cruz, USA
Rabbit ceramide synthase 3	Polyclonal	1:75	Sigma Aldrich
Secondary antibody			
Rhodamine Red-X (Goat anti-rabbit) (IF)		1:300	Jackson immunoresearch Laboratory, USA
Cy3 (Goat Anti-Mouse) (IF)		1:1000	Jackson immunoresearch Laboratory, USA

Table S3: Number of subjects and techniques applied

	Patients	Controls
Study population	n = 20 n= 12 wild type n= 5 heterozygous n= 3 homozygous	n=8
Immunofluorescence	n = 7 n= 4 wild type n= 3 heterozygote	n=3
CER and FFA analysis	n = 20 n= 12 wild type n= 5 heterozygous n= 3 homozygous	n=8
Protein content	n = 20 n= 12 wild type n= 5 heterozygous n= 3 homozygous	n=8
ATR_FTIR	n = 20 n= 12 wild type n= 5 heterozygous n= 3 homozygous	n=8

Supplementary materials and methods

Liquid chromatography-mass spectrometry (LC-MS)

FFA standards ranging from C14-C24 were used to correct for the effect of chain length on the MS response. In addition, the effect of the tape matrix on the MS response was corrected by using the ratio of integrated FFA (C16-30) from extracted human stratum with and without tape strip matrix. As reported earlier, endogenous FFAs C16:0, C17:0, C18:0, C16:1 and C18:1 were also present in the blank tape strips and therefore interfere with the analysis (7). An exogenous compound (358 amu) present only within the tape but not in SC correlated excellently with the FFAs present in the tape (R^2 values range from 0.92-0.98). Using this compound peak, it was possible to calculate the level of exogenous C16:0, C17:0, C18:0, C16:1 and C18:1 in each tape strip and subsequently correct the FFA profile for these levels. The FFA analysis was performed using Target Lynx software version 4.1.

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